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(71) Applicants: THE BOARD OF REGENTS ACTING FOR AND ON BEHALF OF THE UNIVERSITY OF MICHIGAN [US/US]; Ann Arbor, MI 48109 (US). UNIVERSITY OF CONNECTICUT [US/US]; Storrs, CT 06269 (US). ALBERT EINSTEIN COLLEGE OF MEDICINE, A DIVISION OF YESHIVA UNIVERSITY [US/US]; 1300 Morris Park Avenue, Bronx, NY 10461 (US).

(72) Inventors: WILSON, James, M. ; 3686 River Pines Drive, Ann Arbor, MI 48103 (US). GROSSMAN, Mariann ; 834 West Huron Street, #1, Ann Arbor, MI 48103 (US). WU, Catherine, H. ; 15 Rundelane, Bloomfield, CT 06002 (US). CHOWDHURY, Namita, Roy ; 139 Woodhollow Lane, New Rochelle, NY 10804 (US). WU, George, Y. ; 15 Rundelane, Bloomfield, CT 06002 (US). CHOWDHURY, Jayanta, Roy ; 139 Woodhollow Lane, New Rochelle, NY 10804 (US).

(74) Agents: DeCONTI, Giulio, A., Jr. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

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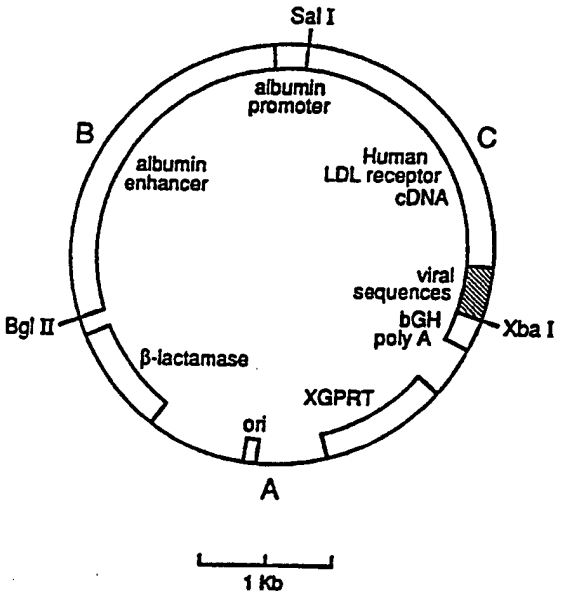
(54) Title: TARGETED DELIVERY OF GENES ENCODING CELL SURFACE RECEPTORS

(57) Abstract

Molecular complexes for targeting a gene encoding a cell surface receptor to a specific cell *in vivo* and obtaining expression of the gene and insertion of the gene-encoded receptor in the cell membrane. An expressible gene encoding a desired cell surface receptor is complexed with a carrier of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent is specific for a cellular surface structure which mediates internalization of ligands by endocytosis. An example is the asialoglycoprotein receptor of hepatocytes. The gene-binding agent is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within a cell. The molecular complex is stable and soluble in physiological fluids and can be used in gene therapy to selectively transfect cells *in vivo* to provide for production, membrane insertion and function of a cellular surface receptor.



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TARGETED DELIVERY OF GENES ENCODING CELL SURFACE RECEPTORS

Background of the Invention

Familial hypercholesterolemia (FH) is an inherited disease in humans, caused by a deficiency of low-density lipoprotein (LDL) receptors. FH is associated with hypercholesterolemia and premature development of coronary heart disease. J.L. Goldstein and M.S. Brown in The Metabolic Basis of Inherited Disease (C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle eds.) McGraw-Hill, New York, Sixth edition, pp. 1215-1250. Current therapies for FH primarily attempt to decrease serum LDL cholesterol by increasing hepatic expression of the LDL receptor. J.L. Goldstein and M.S. Brown in The Metabolic Basis of Inherited Disease supra. This has been accomplished in homozygous patients by orthotopic liver transplantion from donors who express normal levels of LDL receptor. Short term metabolic efficacy in the animal model for FH, the Watanabe Heritable Hyperlipidemic rabbit (WHHL), has been demonstrated by transplanting allogenic wild-type hepatocytes or WHHL hepatocytes genetically

corrected ex vivo with recombinant retroviruses. J.M. Wilson et al. Proc. Natl. Acad. Sci. 85:4421 (1988) and J.M. Wilson et al. Proc. Natl. Acad. Sci. 87:8437 (1990).

The clinical utility of ex vivo gene therapy in the liver may be limited, in part, by the morbidity of the invasive procedures used to harvest and transplant hepatocytes. Delivery of genes to hepatocytes and other cells in vivo would be of value in treating FH and other acquired and inherited diseases.

Summary of the Invention

This invention pertains to a soluble molecular complex for targeting a gene encoding a cell surface receptor, such as the LDL receptor, to a specific cell in vivo and obtaining expression of the gene by the targeted cell. The molecular complex comprises an expressible gene encoding a desired cell surface receptor complexed with a carrier which is a conjugate of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent is specific for a cellular surface structure, typically a receptor, which mediates internalization of bound ligands by endocytosis, such as the asialoglycoprotein receptor of hepatocytes. The cell-specific binding agent can be a natural or synthetic ligand (for example, a protein, polypeptide, glycoprotein, etc.) or it can be an antibody, or an analogue thereof, which specifically binds a cellular surface structure which then mediates internalization of the bound complex. The gene-binding component of the conjugate is a compound

such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within the cell.

The complex of the gene and the carrier is stable and soluble in physiological fluids. It can be administered in vivo where it is selectively taken up by the target cell via the surface-structure-mediated endocytotic pathway. The incorporated gene is expressed and the gene-encoded receptor is processed and inserted into the cell membrane of the transfected cell.

The soluble molecular complex of this invention can be used to specifically transfect cells in vivo to provide for expression of a desired cell surface receptor. This selective transfection is useful for gene therapy and in other applications which require selective genetic alteration of cells to produce a desired surface receptor. In gene therapy, a normal gene can be targeted to a specific cell to correct or alleviate an inherited or acquired abnormality involving a cell surface receptor, such as familial hypercholesterolemia, caused in part by a defect in the LDL receptor gene.

Brief Description of The Figures

Figure 1 shows the structure of the LDL receptor expression vector p9-12alb(h)LDLR. The vector contains the structural gene for human LDL receptor driven by rat albumin promoter and mouse albumin enhancer sequences.

Figure 2 shows that the LDLR-complex is primarily targeted to the liver. ^{125}I uptake by various organs was determined following injection of radiolabeled LDLR-complex.

Figure 3 shows the cellular distribution of the LDLR-complex which indicates the complex is predominantly taken up by hepatocytes.

Figure 4A shows DNA blot hybridization analysis of total cellular DNA which indicates that the LDLR-complex remains intact extracellularly and delivers functional DNA.

Figure 4B shows RNase protection analysis which confirms the presence of recombinant human LDL receptor transcripts in the liver.

Figure 5 shows the results of a two treatment crossover study measuring total serum cholesterol to confirm the presence of human LDL receptor.

Detailed Description of the Invention

A soluble, targetable molecular complex is used to selectively deliver a gene encoding a cell surface receptor to a target cell or tissue in vivo. The molecular complex comprises the receptor-encoding gene to be delivered complexed to a carrier made up of a binding agent specific for the target cell and a gene-binding agent. The complex is selectively taken up by the target cell and the gene-encoded receptor is expressed, processed, and inserted into the cellular membrane.

The gene, generally in the form of DNA, encodes the desired cell surface receptor. Typically, the gene comprises a structural gene encoding the receptor in a form suitable for processing by the

target cell. For example, the gene enc des appropriate signal sequences which direct processing and membrane insertion of the receptor. The signal sequence may be the natural sequence of the receptor or exogenous sequences. The structural gene is linked to appropriate genetic regulatory elements required for expression of the gene-encoded receptor by the target cell. These include a promoter and optionally an enhancer element operable in the target cell. The gene can be contained in an expression vector such as a plasmid or a transposable genetic element along with the genetic regulatory elements necessary for expression of the gene and production of the gene-encoded product.

The carrier component of the complex is a conjugate of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent specifically binds a cellular surface structure which mediates internalization by, for example, the process of endocytosis. The surface structure can be a protein, polypeptide, carbohydrate, lipid or combination thereof. It is typically a surface receptor which mediates endocytosis of a ligand. Thus, the binding agent can be a natural or synthetic ligand which binds the receptor. The ligand can be a protein, polypeptide, glycoprotein or glycopeptide which has functional groups that are exposed sufficiently to be recognized by the cell surface structure. It can also be a component of a biological organism such as a virus, cells (e.g., mammalian, bacterial, protozoan) or artificial carriers such as liposomes.

The binding agent can also be an antibody, or an analogue of an antibody such as a single chain antibody which binds the cell surface structure.

Ligands useful in forming the carrier will vary according to the particular cell to be targeted. For targeting hepatocytes, glycoproteins having exposed terminal carbohydrate groups such as asialoglycoprotein (galactose-terminal) can be used, although other ligands such as polypeptide hormones may also be employed. Examples of asialoglycoproteins include asialoorosomucoid, asialofetuin and desialylated vesicular stomatitis virus. Such ligands can be formed by chemical or enzymatic desialylation of glycoproteins that possess terminal sialic acid and penultimate galactose residues. Alternatively, asialoglycoprotein ligands can be formed by coupling galactose terminal carbohydrates such as lactose or arabinogalactan to non-galactose bearing proteins by reductive lactosamination.

For targeting the molecular complex to other cell surface receptors, other types of ligands can be used, such as mannose for macrophages, mannose-6-phosphate glycoproteins for fibroblasts, intrinsic factor-vitamin B12 for enterocytes and insulin for fat cells. Alternatively, the cell-specific binding agent can be a receptor or receptor-like molecule, such as an antibody which binds a ligand (e.g., antigen) on the cell surface. Such antibodies can be produced by standard procedures.

The gene-binding agent complexes the gene to be delivered. Complexation with the gene must be sufficiently stable in vivo to prevent significant uncoupling of the gene extracellularly prior to

internalization by the targ t cell. However, the complex is cleavable under appropriate conditions within the cell so that the gene is released in functional form. For example, the complex can be labile in the acidic and enzyme rich environment of lysosomes. A noncovalent bond based on electrostatic attraction between the gene-binding agent and the gene provides extracellular stability and is releasable under intracellular conditions.

Preferred gene-binding agents are polycations that bind negatively charged polynucleotides. These positively charged materials can bind noncovalently with the gene to form a soluble, targetable molecular complex which is stable extracellularly but releasable intracellularly. Suitable polycations are polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines and the like. A preferred polycation is polylysine. Other noncovalent bonds that can be used to releasably link the expressible gene include hydrogen bonding, hydrophobic bonding, electrostatic bonding alone or in combination such as, anti-polynucleotide antibodies bound to polynucleotide, and strepavidin or avidin binding to polynucleotide containing biotinylated nucleotides.

The carrier can be formed by chemically linking the cell-specific binding agent and the gene-binding agent. The linkage is typically covalent. A preferred linkage is a peptide bond. This can be formed with a water soluble carbodiimide as described by G. Jung et al. Biochem. Biophys. Res. Commun. 101:599-606 (1981). An alternative linkage is a disulfide bond.

The linkage reaction can be optimized for the particular cell-specific binding agent and gene-binding agent used to form the carrier. Reaction conditions can be designed to maximize linkage formation but to minimize the formation of aggregates of the carrier components. The optimal ratio of cell-specific binding agent to gene-binding agent can be determined empirically. When polycations are used, the molar ratio of the components will vary with the size of the polycation and the size of the gene. In general, this ratio ranges from about 10:1 to 1:1, preferably about 5:1. Uncoupled components and aggregates can be separated from the carrier by molecular sieve chromatography.

The gene encoding the desired cell surface receptor can be complexed to the carrier by a stepwise dialysis procedure. In a preferred method, for use with carriers made of polycations such as polylysine, the dialysis procedure begins with a 2M NaCl dialyzate and ends with a 0.15M NaCl solution. The gradually decreasing NaCl concentration results in binding of the DNA to the carrier.

The molecular complex can contain more than one copy of the same gene or one or more different genes. Preferably, the ratio of polynucleotide to the carrier is from about 1:5 to 5:1, preferably about 1:2.

The molecular complex of this invention can be administered parenterally. Preferably, it is injected intravenously. The complex is administered in solution in a physiologically acceptable vehicle.

Cells can be transfected in vivo for transient expression and production of the gene product. For prolonged expression and production, the gene can be administered repeatedly. Alternatively, the transfected target cell can be stimulated to replicate by surgical or pharmacological means to prolong expression of the incorporated gene. See, for example, U.S. Patent Application Serial No. 588,013, filed September 25, 1990, the teachings of which are incorporated by reference herein.

The method of this invention can be used in gene therapy to selectively deliver a gene encoding a cell surface receptor to a target cell in vivo for expression. A gene encoding a desired cell surface receptor can be targeted to a cell which normally expresses the receptor but which lacks the capacity to produce the receptor or produces an insuffient amount of the receptor because of an acquired or inherited defect. For example, a normal gene can be targeted to a specific cell to correct or alleviate a metabolic or genetic abnormality caused by an inherited or acquired defect in a corresponding endogenous gene encoding a cell surface receptor. Alternatively, the gene can be delivered to a cell which does not normally express the surface receptor to confer a new function upon the cell.

The cell surface receptor can be a receptor for a natural ligand such as a metabolite, a hormone, a growth factor, a cytokine, an ion (ion transport protein), a virus, or a protozoan. It can also be a receptor that mediates cell-cell interaction.

In vivo gene transfer has several potential advantages over organ/cell transplantation in the treatment of metabolic diseases of the liver. One advantage is that the therapeutic gene is expressed in situ in a cell and organ that has not been manipulated ex vivo. In addition, the capacity of this approach to reconstitute hepatic gene expression is theoretically greater than the capacity of cellular therapies which are usually limited by the number of cells that will engraft. Finally, the therapeutic gene can be delivered to the appropriate cell in a noninvasive way with little apparent morbidity.

Familial hypercholesterolemia is an inherited disease in humans caused by a deficiency in the receptor for LDL. In a preferred embodiment, the gene encoding the human LDL receptor is complexed to a conjugate of an asialoglycoprotein and a polycation. The resulting soluble complex is administered parenterally to the individual afflicted with the LDL receptor deficiency in amounts sufficient to selectively transfect cells and to provide sufficient production of the cell surface receptor to attain normal levels of serum cholesterol. DNA encoding human LDL receptor is described in U.S. Patent 4,745,060, the contents of which are incorporated herein by reference.

This invention is illustrated further by th following Exemplification.

Exemplification

An asialoglycoprotein-polycation conjugate consisting of asialoorosomucoid (ASOR) coupled to polylysine, was used to form a soluble DNA complex capable of specifically targeting hepatocytes via asialoglycoprotein receptors present on these cells. The DNA comprised a plasmid, p9-12alb(h)LDLR, containing the structural gene for the human LDL receptor driven by rat albumin promoter and mouse albumin enhancer elements.

Animals

The efficacy of intravenous administration of the LDLR-complex was studied in an animal model of familial hypercholesterolimia, the Watanabe Heritable Hyperlipidemic (WHHL) rabbit. WHHL rabbits were derived from mating homozygous LDL-receptor deficient rabbits and were purchased from Dr. Mahlan at New York University. Wild-type New Zealand White (NZW) rabbits were purchased from Dutchland Farms (Denver, PA). Animals were maintained on a Purina laboratory rabbit chow and weighed 2-3 kg at the time of experimentation. Phlebotomies were performed at 3 to 4 PM. Experiments were conducted in accordance with the guidelines of the Committee on Use and Care of Animals from the University of Michigan and Albert Einstein College of Medicine.

Construction of DNA Vector

A vector capable of expr ssing normal human LDL receptor was constructed (p9-12alb(h)LDLR) for in vivo gene transfer experiments in WHHL rabbits (Figure 1). Transcriptional elements from the mouse albumin gene were used to drive expression of a full-length cDNA for the human LDL receptor. L.E. Babiss et al. Proc. Natl. Acad. Sci. 83:6504 (1986); C.A. Pinkert et al. Genes Dev. 1:268 (1987); K.S. Zaret et al. Proc. Natl. Acad. Sci. 85:9076 (1988); and R.S. Herbat et al. Proc. Natl. Acad. Sci. 86:1553 (1989).

The expression vector was constructed in a single three-part ligation using fragments that were cloned in a directional manner (Figure 1). The three fragments used in this construction are as follows: Fragment A is an XbaI to BglII fragment (3.6 kb) of plasmid MTEV.JT (provided by J. Trill) which contains several functional elements including a 231 base pair (bp) fragment of genomic DNA spanning the polyadenylation signal of the bovine growth hormone gene, ß-lactamase and the prokaryotic origin of replication from PUC 19, and a eukaryotic transcriptional unit expressing xanthine-guanine phosphoribosyltransferase (XGPRT); Fragment B includes sequences spanning an enhancer located 5' to the mouse albumin gene (-12 to -9 kb) excised on an Eco RV to BglII fragment and fused in reverse orientation to sequences spanning the mouse albumin promoter (-282 to +21). L.E. Babiss et al. Proc. Natl. Acad. Sci. 83:6504 (1986); C.A. Pinkert et al. Genes Dev. 1:268 (1987); K.S. Zaret et al. Proc. Natl. Acad. Sci. 85:9076 (1988); and R.S. Herbat et al. Proc. Natl. Acad. Sci. 86:1553

(1989). The 5' end of the chimeric fragment is formed by the natural BglII site of the albumin enhancer (-9 kb) while the 3' end of the fragment contains a synthetic SalI site attached to position +22 of the albumin promoter sequence; and Fragment C is a SalI to XbaI fragment (3.0 kb) derived from a previously published retroviral vector LTR-LDLR. J.M. Wilson et al. Proc. Natl. Acad. Sci. 85:4421 (1988). The LTR-LDLR vector includes the entire cDNA for human LDL receptor (2.6 kb) along with 430 bp of additional 3' sequence derived from the retroviral genome (nucleotides 7816 to 8113 of the Moloney murine leukemia virus genome; see C. Van Beveren et al. in RNA Tumor Viruses (R. Weiss, N. Teich, H. Varmus, and J. Coffin eds.) Cold Spring Harbor Lab., Cold Spring Harbor N.Y., 2nd Ed., pp. 766-783 (1985) for numbering). The 5' end of the LDL receptor cDNA was converted to a SalI site in preparation for this construction.

LDL receptor sequences in p9-12alb(h)LDLR were replaced with sequences encoding the prokaryotic gene chloroamphenicol acetyltransferase (CAT) to generate a vector called p9-12albCAT (provided by S. Camper). This vector was used as a negative control in metabolic experiments based on the assumption that CAT expression does not specifically affect intracellular hepatic cholesterol metabolism. DNA/protein complexes were synthesized with either p9-2alb(h)LDLR (LDLR-complex) or p9-12albCAT (CAT-complex) (See below).

Construction of Gene Carrier and Gene Carrier Complexes

A high affinity ligand for the asialoglycoprotein receptor, asialoorosomucoid (ASOR), was covalently attached to polylysine to produce a gene carrier useful in the study of the organ and cellular distribution of DNA/protein complex uptake in vivo. In order to introduce a radioisotope into the DNA/protein complex without perturbing its structural configuration LDLR-complexes were synthesized with ^{125}I-labeled ASOR. The gene carriers were prepared as described previously. G.Y. Wu et al. J. Biol. Chem. 264:16985 (1989).

Briefly, human orosomucoid isolated from pooled human plasma, was desialyted with neuraminidase and subsequently coupled to poly L-lysine (MW=59,000), using 1-ethyl(-3-)-3-dimethyl amino propyl carbodiimide. The ASOR/poly L-lysine conjugate was purified by molecular sieve chromatography as described previously and complexed to plasmid DNA using an agarose gel retardation assay to determine optimal conjugate to DNA ratios for each plasmid. G.Y. Wu et al. J. Biol. Chem. 264:16985 (1989). Large scale complexes were made by successive stepwise dialysis of conjugate-DNA mixtures 18 hours each against 2 M, 1.5 M, 1.0 M, 0.5 M. and finally 0.2 M NaCl. Final samples were filtered through 0.45 μ membranes and checked by agarose gel electrophoresis for the presence of protein/DNA complex and the absence of free DNA. ASOR was labeled with ^{125}I (G.Y. Wu and C.H. Wu J. Biol. Chem. 262:4429 (1987); and G.Y. Wu and C.H. Wu Biochemistry 27:887 (1988)),

conjugated to poly L-lysine, and the radiolabeled ASOR/poly L-lysine conjugate was complex d with p9-12alb(h)LDLR plasmid as described above.

Targeting of the LDLR-Complex to the Liver

Animals were anesthetized with ketamine HCl (40 mg/kg) and xylazine (10 mg/kg) in preparation for the experiment. Radiolabeled LDLR-complexes were injected into the marginal ear vein of New Zealand White (NZW) or WHHL adult rabbits (2 to 3 kg) which were euthenized and exanguinated 10 minutes later. Individual organs were harvested and analyzed for incorporation of radioactivity (Figure 2). DNA/protein complex was rapidly cleared from the plasma and primarily taken up by the liver (85% of total recovered radioactivity) after 10 minutes. Organ distribution of uptake was shown to be independent of the phenotype of the recipient animal (NZW, heterozygous WHHL, and homozygous WHHL) and the dose of injected complex (from 0.17 mg to 4 mg of DNA in a complex).

The average recovered radioactivity for the four animals presented in Figure 2 was estimated to be 99 +/-10 percent (mean +/- 1 S.D., N=4). Data are presented as % recovered radioactivity in individual organs including liver, spleen, lung, heart, kidney, and blood. (A) Tracer quantities of ASOR labeled complex (0.17 mg of DNA; specific activity = 2×10^6 cpm/μg) into a WHHL rabbit (homozygous). (B) Tracer quantities of ASOR labeled complex into a WHHL rabbit (heterozygous). (C) Tracer quantities of ASOR labeled complex (0.17 mg of DNA; specific activity = 2×10^6 cpm/μg) into a NZW rabbit. (D) Therapeutic

quantities f ASOR labeled complex (4.0 mg of DNA; specific activity = 0.85 x 10^5 cpm/μg) into a WHHL rabbit (homozygous).

Cellular Distribution of the LDLR-Complex

Although the hepatocyte is the predominant cell type in the liver and the likely target for gene transfer, nonparenchymal cells of the liver, such as endothelial cells and kupffer cells, could potentially serve as alternative targets. To visualize the cellular distribution of LDLR-complex, rabbits were injected with radiolabeled LDLR-complex as described above. Ten minutes after injection, the portal vein was cannulated, and the liver was perfused with 154 mM NaCl for 5 minutes followed by Karnovsky's fixative for 10 minutes. R. St. Hilaire et al. Proc. Natl. Acad. Sci. 80:3797 (1983). Random blocks of liver were cut on ice to 2 - 5 mm^3 cubes and were kept in fresh fixative for 2 hours prior to transfer to a 0.2 M sodium bicarbonate, pH 7.4, overnight at room temperature. Blocks were embedded in Epox 812 (W.O. Dobbins III in Diagnostic Electron Microscopy (B.F. Trump and R.T. Jones eds.) Wiley, New York, Vol. 1, pp. 253-339) and 1 μm sections were cut and mounted on polylysine-coated slides which were dipped in NTB3 nuclear emulsion. After exposure for 2 - 3 weeks at 4° C, slides were developed with Kodak D-19, and stained with 0.5% toluidine blue in 1% sodium benzoate (See Figure 3). Panel A: Analysis of liver tissue from a WHHL that received ASOR labeled complex (4.0 mg of DNA; specific activity = 0.85 x 10^5 cpm/μg). Panel B: Analysis of liver tissue from a WHHL rabbit that was injected with saline. In each case, magnification is 400x.

Radioactive signal, detected as silver grains, was 100-fold over that observed in unlabeled liver tissue (Figure 3). The majority of this signal (>90%) was seen as discrete grains located over hepatocytes (Figure 3A). Radioactive signal was infrequently seen over kupffer cells as aggregates or discrete grains (Figure 3A). This nonparenchymal cell uptake may represent larger forms of the DNA/protein complex that have been phagocytized by kupffer cells. These studies indicate that the DNA/protein complex is rapidly taken up by hepatocytes after injection into peripheral blood.

Intracellular Distribution of the LDLR-Complex

The intracellular fate of the recombinant gene was studied in WHHL rabbits after administration of unlabeled LDLR-complex. Animals were euthanized 10 minutes, 4 hours, and 24 hours after in vivo gene transfer and liver tissue was characterized with respect to the abundance and structural integrity of the recombinant gene as well as the level of recombinant derived RNA.

DNA Blot Hybridization Studies:

Total cellular DNA isolated from liver of WHHL rabbits was analyzed for the presence of p9-12alb(h)LDLR DNA sequences by blot hybridization (Figure 4A). J.M. Wilson et al. Proc. Natl. Acad. Sci. 87:8437 (1990). DNA was restricted with Bam HI to excise a 1.4 kb fragment from the plasmid, fractionated by agarose gel electrophoresis, and transferred to Zetabind. Filters were hybridized with a vector specific restriction fragment (Hind III to Eco RI fragment of 3Z-env) that had been ^{32}P

labeled to high specific activity. J.M. Wilson et al. Proc. Natl. Acad. Sci. 85:4421 (1988). Samples include control WHHL DNA (10 µg) mixed with the equivalent of 100 copies/cell (750 pg; lane '100c'), 10 copies/cell (75 pg; lane '10c'), and 1 copy/cell (7.5 pg; lane '1c') of p9-12alb(h)LDLR plasmid DNA. Additional DNA samples (10 µg) are from WHHL rabbits injected with LDLR-complex and harvested 10 minutes (lane '10 min'), 4 hours (lane '4 hr'), and 24 hours (lane '24 hr') later. The filter was exposed to film for 45 minutes (top panel) and 24 hours (bottom panel). Molecular weight markers are noted in base pairs along the left border.

The results indicate that liver tissue harvested 10 minutes after injection of the LDLR-complex demonstrated very high levels of the intact fragment along with some partially degraded plasmid DNA; comparison to plasmid controls indicated that this tissue contained approximately 5,000 to 10,000 copies of plasmid per cell. This estimate of gene targeting is in agreement with the level of gene delivery to the liver expected from the amount of DNA injected (this estimate is based on the injection of 4 mg of plasmid (10 kb in size) into a 2-3 kg rabbit that has $2 - 4 \times 10^{10}$ hepatocytes). Analysis of livers harvested at later time points revealed ongoing degradation of the plasmid and a progressive decline in the abundance of the intact plasmid from 100 copies/cell at 4 hours to 1 copy/cell at 24 hours. These experiments indicate that the LDLR-complex remains intact in the blood and capable of efficient delivery of large quantities of DNA to the liver. The internalized plasmid DNA is eventually degraded.

RNase Protection Assays:

Liver tissu s were analyzed for the presence and abundance of recombinant human LDL receptor transcripts using a quantitative RNase protection assay. J.M. Wilson et al. Proc. Natl. Acad. Sci. 87:8437 (1990). RNA derived from the p9-12alb(h)LDLR vector was detected with an antisense RNA probe that is synthesized from the previously described vector 3Z-env. J.M. Wilson et al. Proc. Natl. Acad. Sci. 87:8437 (1990). This RNA probe is complementary to vector specific sequences in the 3' untranslated region of the recombinant transcript. RNase protection of the resulting duplex produces a protected fragment of 172 bp (Figure 4B). Antisense RNA that specifically detects endogenous WHHL LDL receptor RNA was used as an internal control in each assay.

The transcription vector used to synthesize the RNA probe (3Z-wLDLR) was constructed in the following manner. A restriction fragment from a WHHL LDL receptor cDNA clone, spanning the Sma I site at position 211 to the Nar I site at 514, was isolated (See T. Yamamoto et al. Science 232:1230 (1986) for numbering). The Nar I site was converted to a Hind III site with synthetic linkers and the revised fragment was ligated with the Hind III to Sma I backbone fragment of pGEM3Zf(+) (Promega). Endogenous WHHL LDL receptor RNA is detected with the 3Z-wLDLR probe in RNase protection assays as a 80 bp band. Transcription vectors were linearized with Eco RI (3Z-env) or Mnl I (3Z-wLDLR) and used as templates in transcription reactions according to the recommendations of the manufacturer (Promega). RNA

probes were gel purified prior to use. M.H. Finer in Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Techniques (E.J. Murray and J.M. Walker eds.) The Humana Press Inc., Clifton, NJ, pp. 1-15 (1990).

Total cellular RNA prepared from liver was hybridized with equal quantities of 3Z-env and 3Z-wLDLR probe ($5x10^5$ cpm of each probe per assay) and analyzed for protection to digestion with RNase A. J.M. Wilson et al. Proc. Natl. Acad. Sci. 87:8437 (1990). Samples were electrophoresed through a 6% polyacrylamide/urea denaturing gel; a representative autoradiograph is presented. Radioactivity in the resulting bands was quantified with a Beta Scope 630 (Betagen, Waltham MA). The following samples were analyzed. Lane '3T3' contains RNA (100 µg) from a control WHHL supplemented with RNA (100 ng) from a fibroblast cell line (NIH3T3) that produces recombinant LDL receptor transcripts containing sequences complementary to the 3z-env probe. Additional samples include RNA (100 µg) from an untransfected WHHL rabbit (lane 'Mock') and from WHHL rabbits injected with LDLR-complex and harvested 10 minutes (lane '10 min'), 4 hours (lane '4 hr'), and 24 hours (lanes 24A and 24B, representing two different animals). The undigested probes (2×10^3 cpm/lane) were electrophoresed in lanes '3Z-env' and '3Z-wLDLR'. Molecular weight markers (γATP labeled pBr322/ΦX Hae III fragments) and their corresponding sizes in base pairs are presented in the far right lane. The closed arrow indicates the location of the 3Z-env protected band while the open arrow indicates the location of the 3Z-wLDLR protected band.

Endogenous LDL receptor RNA was specifically detected in RNase protection assays as a 80 bp protected fragment; no hybridization to human LDL receptor RNA was noted. The intensity of the resulting bands is proportional to the amount of RNA used in the initial hybridization suggesting that this assay can be used to quantify recombinant and endogenous transcripts.

Recombinant RNA was not detected in mock-transfected liver or in liver harvested 10 minutes after administration of the LDLR-complex. Livers removed 4 hours and 24 hours after in vivo gene transfer contained significant levels of the recombinant transcript. Quantitative analysis of the assay was used to estimate the abundance of vector-derived RNA relative to the endogenous LDL receptor transcript; levels of the recombinant LDL receptor transcript rose from undetectable at 10 minutes (< 0.05% of endogenous), to 1.3% of endogenous at 4 hours, and 4.0% (24A) and 2.0% (24B) of endogenous at 24 hours.

Metabolic Effects of LDLR-Complex

Experiments were performed to determine the metabolic effects of hepatocyte directed gene transfer in vivo. WHHL rabbits were injected with LDLR-complex or CAT-complex and analyzed for changes in total serum cholesterol. Six WHHL rabbits were entered into a protocol that involved a two treatment (injection of p9-12alb(h)LDLR (LDLR-complex) or p9-12albCAT (CAT-complex) plasmid; 5.0 mg of total DNA in a complex/dose), two period, cross-over design, with repeated measurements of total serum

cholesterol within each period (Figure 5). The DNA/protein complex was injected into the marginal ear vein over a 2 minute time period. Animals A-C received LDLR-complex on day 0 and CAT-complex on day 12, whereas animals D-F received CAT-complex on day 0 and LDLR complex on day 12. Venous samples were subsequently obtained through a marginal ear vein and analyzed for total serum cholesterol. P. Trinder Ann. Clin. Biochem. 12:226 (1974). The data were analyzed using a repeated measures analysis of covariance with the mean of the three baseline measurements as the covariate. B. Jones and M.G. Kenward Design and Analysis of Cross-Over Trials, Chapman and Hill, 1989.

Injection of the LDLR-complex led to an immediate but transient decrease in total serum cholesterol by 25 to 30 % of the pretreatment value; this did not occur in animals injected with the CAT-complex. There was a statistically significant time trend on these differences ($p<0.001$) which reached a maximum value at two days after injection and then decreased thereafter, becoming non-significant following the sixth day after injection. No evidence of carry-over from one experiment to another was noted ($p=0.8148$). Areas under the cholesterol time curve were also analyzed. Treatment with LDLR-complex was associated with a highly significant decrease in the area under the cholesterol time curve ($p=0.0035$) compared to injection of the CAT-complex. There was no evidence of any carry-over ($p=0.1625$) or period effects ($p=0.8010$).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

Claims

1. A soluble molecular complex for targeting a gene encoding a cell surface receptor to a specific cell, the complex comprising an expressible gene encoding the cell surface receptor complexed with a carrier of a cell-specific binding agent and a gene-binding agent.

2. A soluble molecular complex of claim 1, wherein the expressible gene is DNA.

3. A soluble molecular complex of claim 1, wherein the expressible gene encodes the human LDL receptor.

4. A soluble molecular complex of claim 1, wherein the gene-binding agent is a polycation.

5. A soluble molecular complex of claim 4, wherein the polycation is polylysine.

6. A soluble molecular complex of claim 1, wherein the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis.

7. A soluble molecular complex of claim 6, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.

8. A soluble mol cular complex of claim 7, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.

9. A soluble molecular complex of claim 1, wherein the expressible gene is complexed with the gene-binding agent by a noncovalent bond.

10. A soluble molecular complex of claim 1, wherein the cell-specific binding agent is linked to the gene-binding agent by a covalent bond.

11. A soluble molecular complex of claim 1, wherein the expressible gene is complexed with the gene-binding agent so that the gene is released in functional form under intracellular conditions.

12. A pharmaceutical composition comprising a solution of the molecular complex of claim 1 and a physiologically acceptable vehicle.

13. A soluble molecular complex for targeting a gene encoding a cell surface receptor to a hepatocyte, the complex comprising an expressible gene encoding the cell surface receptor complexed with a carrier of a ligand for the asialoglycoprotein receptor and a polycation.

14. A soluble molecular complex of claim 13, wherein the expressible gene encodes the human LDL receptor.

15. A soluble molecular complex of claim 13, wherein the polycation is polylysine.

16. A soluble molecular complex of claim 13, wherein the gene is contained in an expression vector along with genetic regulatory elements necessary for expression of the gene by the hepatocyte.

17. A soluble molecular complex of claim 16, wherein the expression vector is a plasmid or viral DNA.

18. A soluble molecular complex for targeting a gene encoding the human LDL receptor to a hepatocyte, the complex comprising an expressible gene encoding the human LDL receptor complexed with a carrier of a ligand for the asialoglycoprotein receptor and a polycation.

19. A soluble molecular complex of claim 18, wherein the polycation is polylysine.

20. A method of delivering an expressible gene encoding a cell surface receptor to a specific cell of an organism for expression by the cell, comprising administering to the organism a soluble molecular complex comprising the expressible gene encoding the cell surface receptor complexed with a carrier of a cell-specific binding agent and a gene-binding agent.

21. A method of claim 20, wherein the expressible gene is DNA.

22. A method of claim 20, wherein the expressible gene encodes the human LDL r ceptor.

23. A method of claim 20, wherein the gene-binding agent is a polycation.

24. A method of claim 23, wherein the polycation is polylysine.

25. A method of claim 20, wherein the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis.

26. A method of claim 25, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.

27. A method of claim 26, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.

28. A method of claim 20, wherein the molecular complex is administered intravenously.

29. A method of selectively transfecting hepatocytes in vivo with a gene encoding a cell surface receptor, comprising intravenously injecting a pharmaceutically acceptable solution of a molecular complex comprising an expression vector containing an expressible gene encoding the cell surface receptor complexed with a carrier of a ligand for the asialoglycoprotein receptor and a polycation.

30. A method of claim 29, wherein the expressible gene encodes the human LDL receptor.

31. A method of claim 29, wherein the polycation is polylysine.

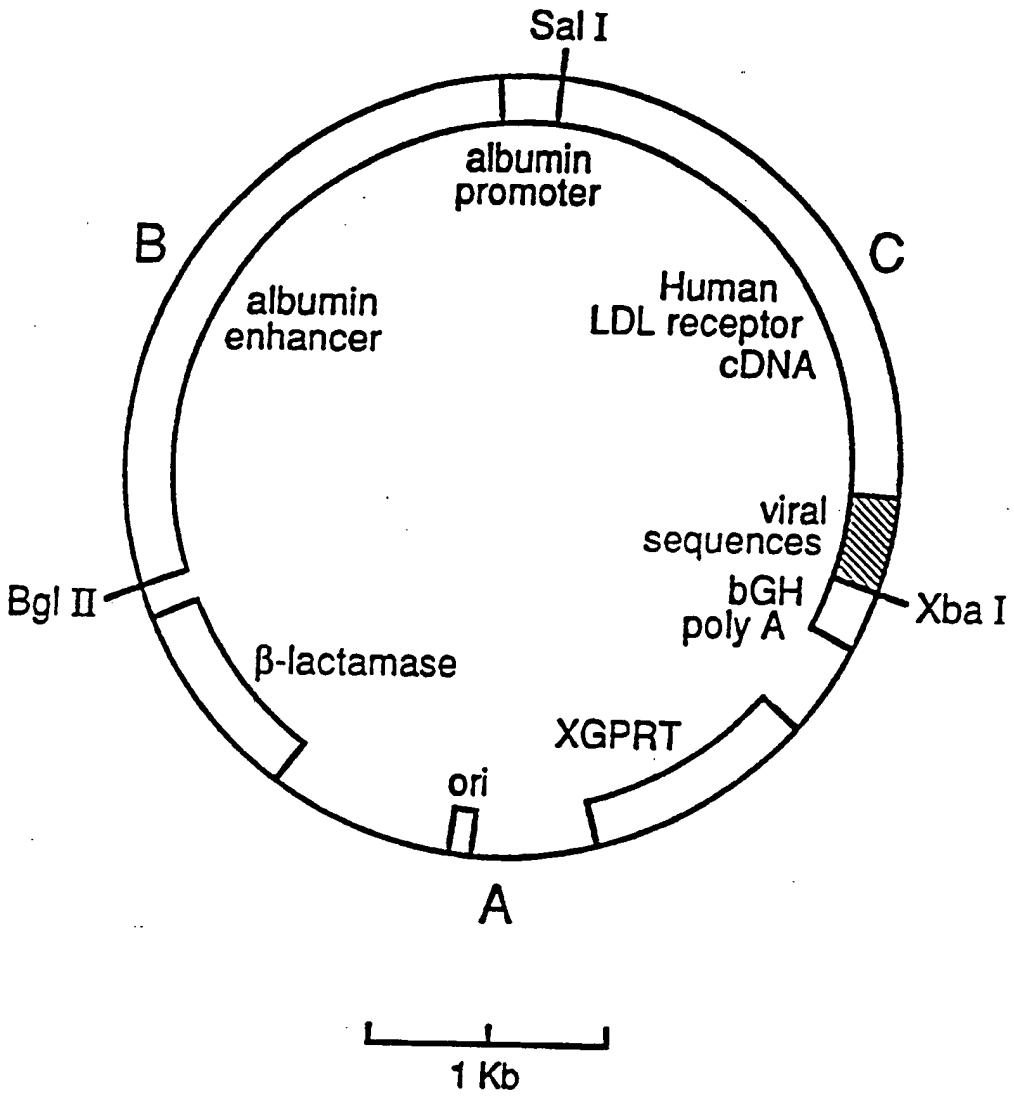

Sal I
albumin promoter
B
C
albumin enhancer
Human LDL receptor cDNA
viral sequences
bGH poly A
Xba I
Bgl II
β-lactamase
XGPRT
ori
A
1 Kb

FIGURE 1

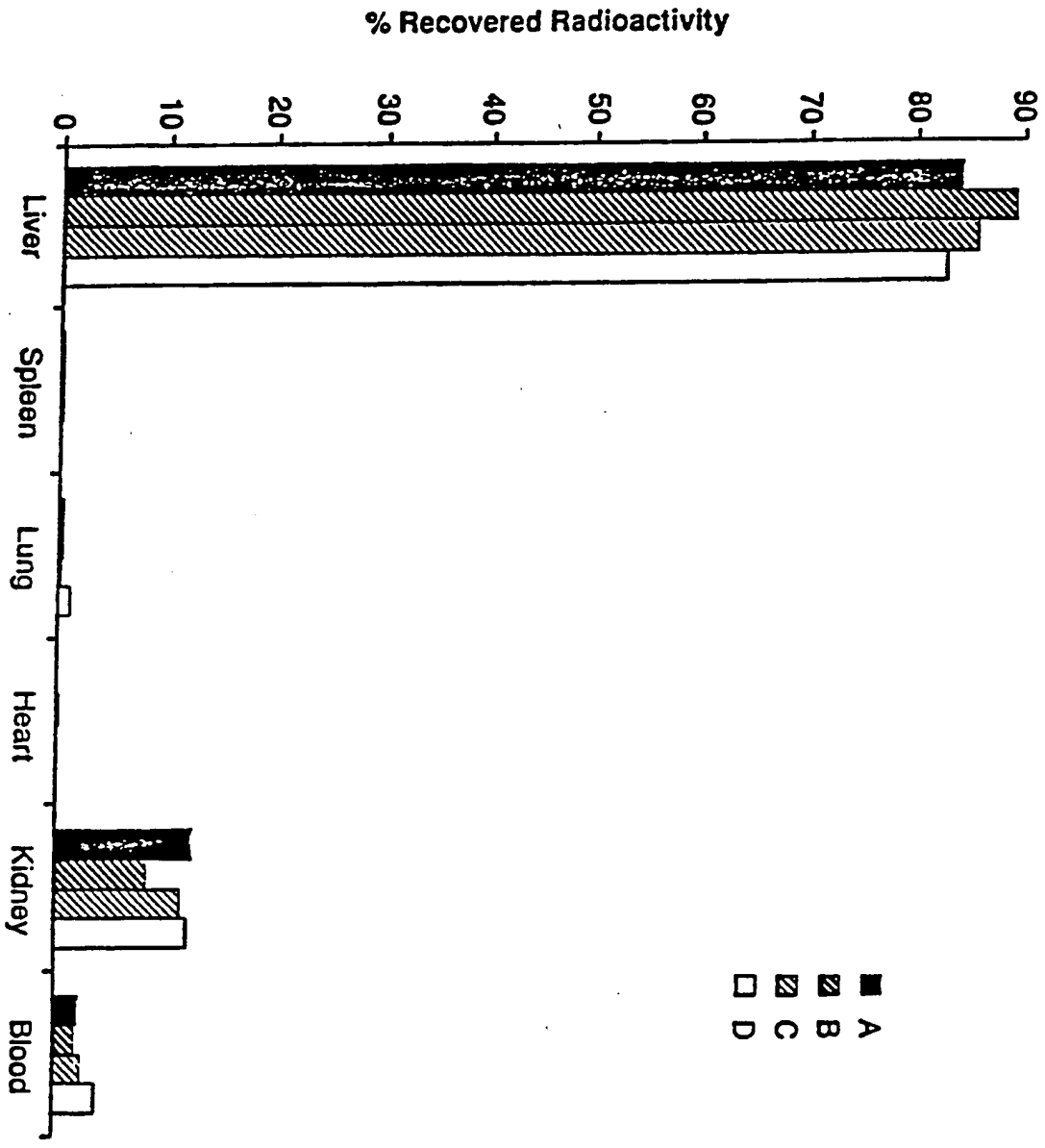
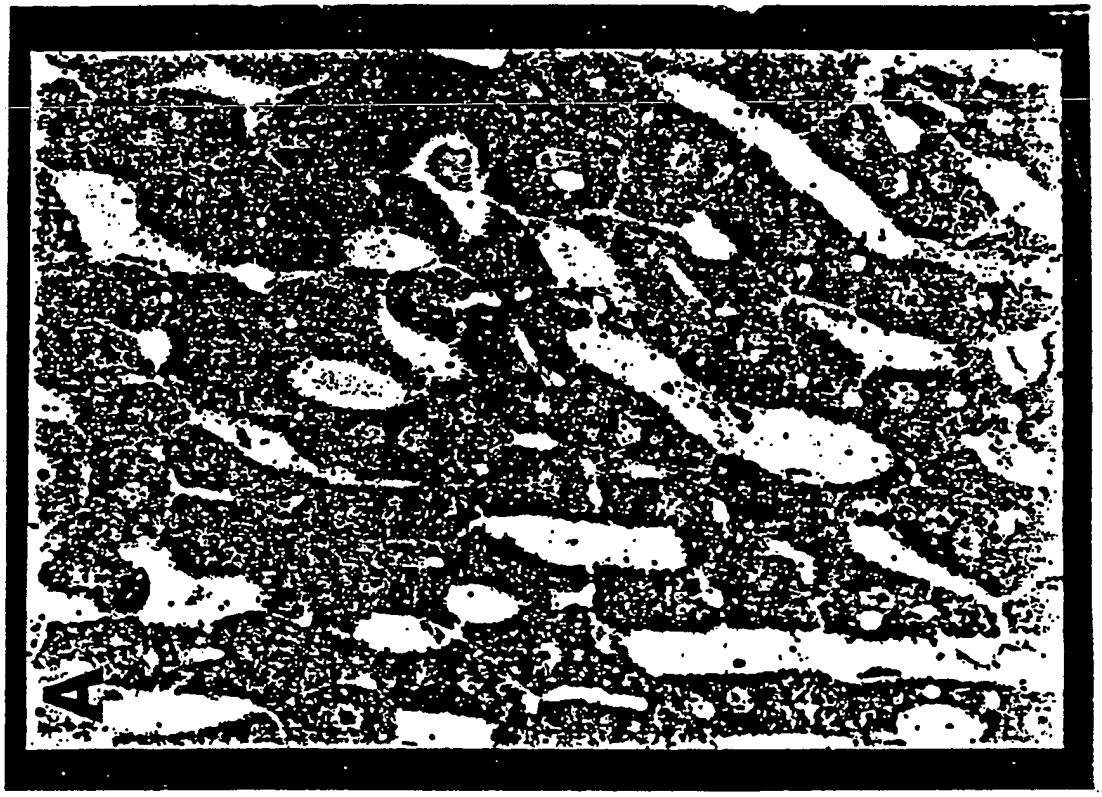

% Recovered Radioactivity
0
10
20
30
40
50
60
70
80
90
Liver
Spleen
Lung
Heart
Kidney
Blood
A
B
C
D

FIGURE 2



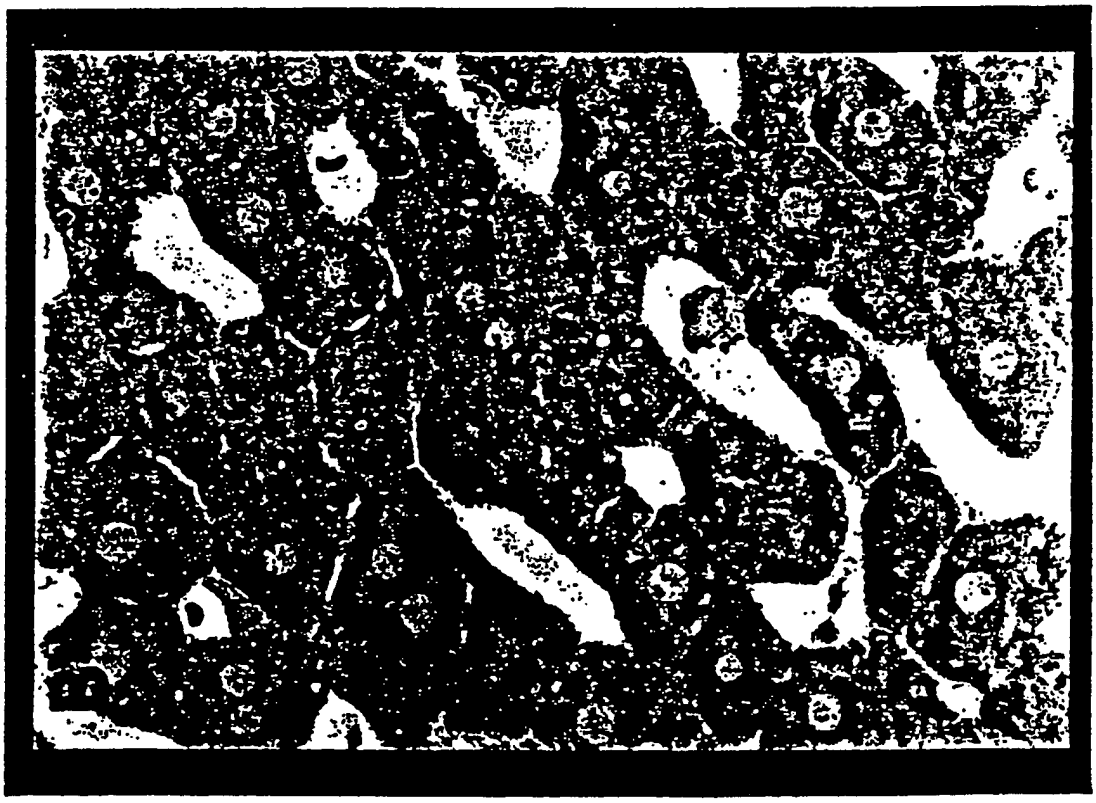
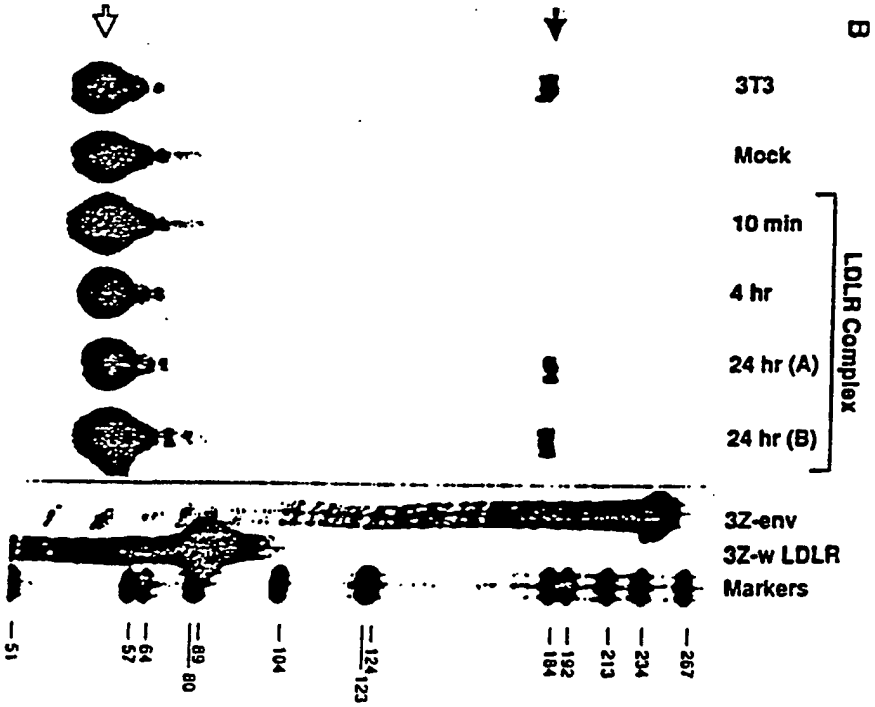


FIGURE 3


B
3T3
Mock
10 min
4 hr
24 hr (A)
24 hr (B)
LDLR Complex
3Z-env
3Z-w LDLR
Markers
51
64
57
89
80
104
124
123
192
184
213
234
267

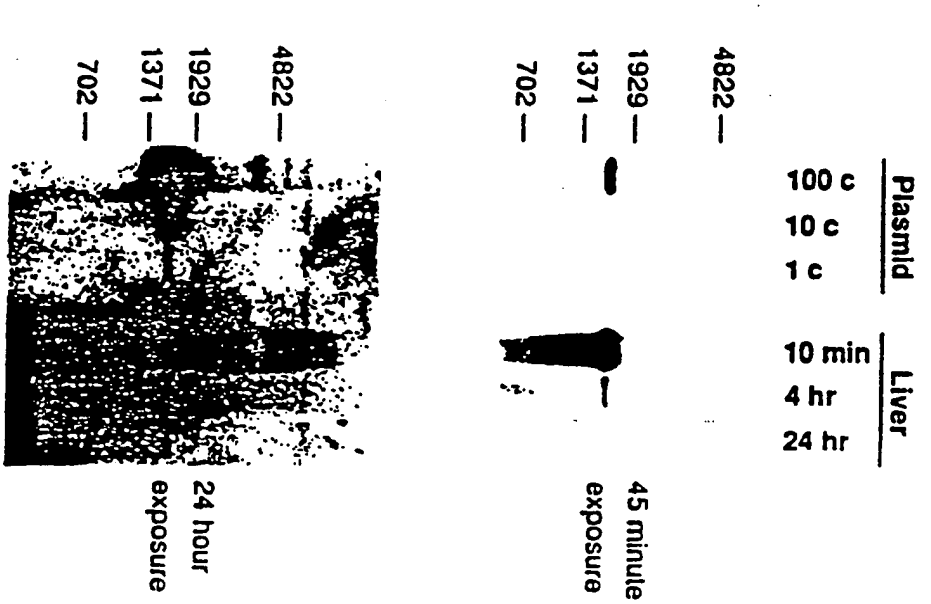

A
4822
1929
1371
702
100 c
10 c
1 c
Plasmid
10 min
4 hr
24 hr
Liver
45 minute exposure
24 hour exposure

FIGURE 4

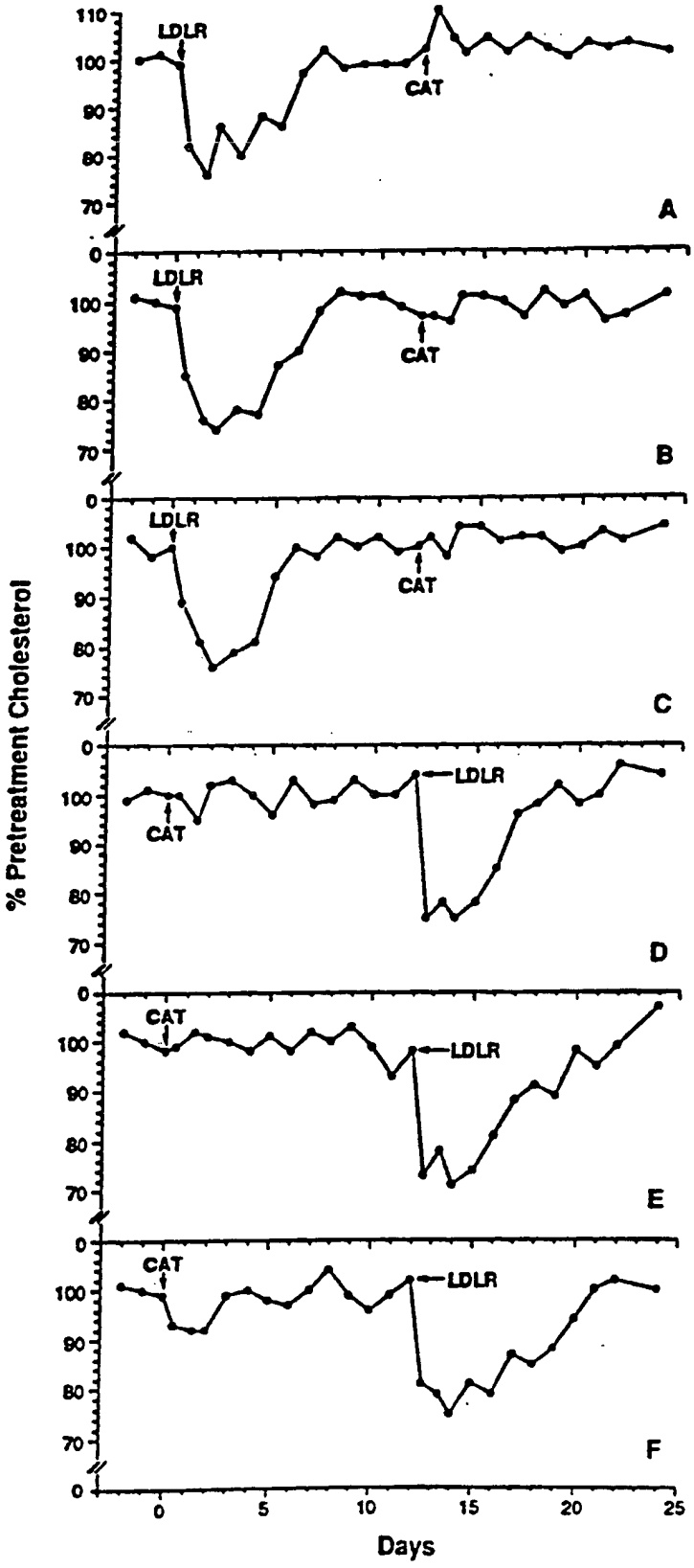

% Pretreatment Cholesterol
LDLR
CAT
A
B
C
D
E
F
110
100
90
80
70
0
0
5
10
15
20
25
Days

FIGURE 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/03639

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/87; C12N15/12; A61K48/00; C07K15/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	CLINICAL RESEARCH vol. 39, no. 2, 9 April 1991, THOROFARE NJ, US page 326A; M. GROSSMAN ET AL.: 'Hepatocyte-directed gene transfer in vivo leads to transient improvement of hypercholesterolemia in LDL receptor deficient rabbits.' see abstract	1-3,6-8, 12-14, 16-18, 20-22, 25-27, 29-30
Y	BIOTHERAPY vol. 3, no. 1, January 1991, DORDRECHT, THE NETHERLANDS pages 87 - 95; G. WU ET AL.: 'Delivery systems for gene therapy.' see abstract	1-27, 29-31

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 SEPTEMBER 1992	29. 09. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	NOOIJ F.J.M.

2

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,8 907 136 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 10 August 1989 see claims	1-27, 29-31
P,Y	SOMATIC CELL AND MOLECULAR GENETICS vol. 17, no. 3, May 1991, NEW YORK pages 287 - 301; D. DICHEK ET AL.: 'Low-density-lipoprotein receptors in the Watanabe heritable hyperlipidemic rabbit.' see abstract	1-27, 29-31
Y	HEPATOLOGY vol. 12, no. 4(2), 1990, NEW YORK, US page 922; J. WILSON ET AL.: 'Targeted gene transfer to hepatocytes in vivo: Mechanism(s) of persistent gene expression.' see abstract 338	1-27, 29-31
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 267, no. 2, 15 January 1992, BALTIMORE MD, US pages 963 - 967; J. WILSON ET AL.: 'Hepatocyte-directed gene transfer in vivo leads to transient improvement of the hypercholesterolemia in low density lipoprotein receptor-deficient rabbits.' see the whole document	1-27, 29-31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03639

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)* incompletely

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 28
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Rule 39.1(iv) - PCT:
Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.
./.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210(2)

Claims 20-27, 29-31 (searched incompletely)

Although claims 20-27 and 29-31 are directed to a method of treatment of the animal/human body, the search has been carried out and based on the alleged effects of the compound/composition.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9203639 SA 60259

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 16/09/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8907136	10-08-89	EP-A- 0400047	05-12-90
		JP-T- 3503718	22-08-91